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SCREENING FOR PRESENCE OF THREE SYNTHETIC PHOSPHODIESTERASE TYPE -5 (PDE-5) INHIBITORS (SILDENFIL, VARDENAFIL AND TADALAFIL) ILLEGALLY ADULTERATED IN NATURAL HERBAL PRODUCTS INTENDED TO BE USE FOR MALE SEXUAL POTENCY IN KUWAIT

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ABSTRACT

Screening studies were conducted to investigate the presence of three synthetic PDE-5-inhibitors, Sildenafil (S), Tadalafil (T) and Vardenafil (V) illegally adulterated in natural herbal products. These herbal products have been a subject for registration by Kuwait Drug and Food Quality Control Administration (KUFDA) as a natural herbal products for improving sexual performance for man in the period from 2003 to 2012. For that purpose an analytical procedure based on liquid chromatography -photodiode array detector combined with electrospray ionization - mass spectrometry [LC—PDA—ESI (+) —MS] has been developed and validated. Extraction for targeted analytes were performed by a mixture of methanol: acetonitrile: water (1:3:2, v/v/v). After sonification for 10 min. and centrifugation at 3000 rpm for 10 min., 10ul from the clear supernatant was injected on symmetry 300 C18 column. Analytes separation were carried out by isocratic elution using mobile phase consisting of methanol: acetonitrile: 1% acetic acid (20:20:60.v/v/v) at a flow rate of 0.4 mL/ min under ambient conditions. Detection was done simultaneously by photdiode array detector (PDA detector) combined with a single quadruple mass analyzer interfaced with electrospray ionization operated in positive ion mode [PDA —ESI (+) —MS]. The method appears specific, sensitive, accurate and relatively simple in both sample preparation and equipments. The procedure providing a very useful tool for rapidly screening (inspection studies) of large amount of samples from herbal products. As examples of adulteration we present the results for presence of at least one of the previously mentioned adulterants during application of the developed method on analysis of 485 samples of tested natural herbal products. The percentage of adulterated samples were ranged from 20.5 to 84.4% within 10 years period. Tadalafil followed by sildenafil presents the main adulterants in the investigated samples. Beginning from year 2010, adulteration (about 25.5-40.5% of analyzed samples) has been done illegally by addition of a mixture of two synthetic PDE-5-inhibitors (S and T). Quantification of S, T and V in positive samples revealed that their doses were sufficiently high to be therapeutic. On the other hand 2-4 % of the positive samples for sildenafil were found contains over therapeutic dose. According to KUFDA rules all adulterated herbal products have been cancelled from registration and banned to be used by men in Kuwait.

KEYWORDS: Adulteration, Synthetic Phosphodiesterase Type-5 Inhibitors, PDE-5, Natural Herbal Products

INTRODUCTION

Over the last few decades there has been an exponential growth in the field of using “natural” herbal products as herbal medicine or dietary supplementary food and nowadays plays an active part in humans health care. These products are regarded by many as being harmless because of their natural origin, and helpful to the treatment of some chronic diseases such as erectile dysfunction (ED) in male (ED is a common important medical disorder [12]) and to maintenance of physical fitness of humans. On the other hand, there are currently three synthetic phosphodiesterase type 5 (PDE-5) inhibitors, sildenafil citrate (brand name Viagra), vardenafil hydrochloride) (brand name Levitra) and tadalafil (brand name Cialis) that have been evaluated as an orally effective drugs for the treatment of ED [6, 7, 24 27, 31 and 32]. Adulterations of “natural” herbal products with at least one of the previously mentioned synthetic PDE-5 inhibitors has been reported since 2001 [1, 2, 5, 8, 9, 13, 16, 17, 18, 19, 28, 30, 33, 35 and 45]. It is true that adulteration of “natural” herbal products with undeclared synthetic PDE-5 inhibitors is a growing trend and poses a health threat to consumers. It is important to note that synthetic PDE-5 inhibitors are prescription drugs and should be taken only under the supervising of a physician; because of their side effects which can be include headache, dyspepsia, flushing and colour visual disturbances [3]. Studies by [26] indicated that interaction between sildenafil (PDE-5 inhibitor) and certain prescription drugs containing nitrates (such as nitroglycerin) may drastically lower blood pressure of the patients to dangerous level. As nitrate medications are commonly used by the patients with diabetes, hypertension, hyperlipidemia and ischemic heart disease, these patients should not take PDE -5 inhibitors. Since the use of PDE-5 inhibitors is contraindicated in these patients, some may look to alternative medicines such as “natural” herbal products for ED treatment. So the possibility of an individual taking nitrate in combination with herbal products that have been adulterated with synthetic PDE-5 inhibitors, may have serious health consequences.

In order to prohibit the marketing of those illegally adulterated “natural “ herbal products and save human health in Kuwait, the Kuwaiti Food and Drug Administration (KUFDA) has been conducting thorough ongoing inspection on “natural” herbal products for the presence of S, T and V in these products. Therefore it was necessary to develop an analytical method that can selectively and simultaneously be used to screen for the presence of these adulterants in herbal products before their purchasing on the commercial market and consumption by humans in Kuwait. Since 2001, the reported methods concerning detection and determination of S, T, V as adulterants in herbal products and dietary supplements were mainly, LC—PDA, LC—PDA — ESI (+) —MS and LC— PDA —ESI — (+) —MS/ MS [1, 2, 5, 8, 9, 13, 16, 17, 18, 19, 28, 30, 33, 35 and 45]. Another analytical technique such as NMR, IR, FTIR and MS have been carried out in a few studies for structural identification on isolated compounds [2 and 13]. From all these studies it can be concluded that the use of, LC—PDA — ESI (+) —MS (because of its selectivity and sensitivity) is becoming increasingly popular in the field of analysis of undeclared synthetic PDE –5 inhibitors adulterated in the “natural“ herbal products.

The aim of this work was to conduct a screening studies to investigate the presence of three synthetic PDE-5 inhibitors [sildenafil (S), vardenafil (V) and tadalafil (T)] as adulterants in natural herbal products. These herbal products have been a subject for registration by KUFDA in the period from 2003 to 2012 as a natural product (pre-market herbal samples advertised usually as a natural) for improving sexual performance for man. For that purpose an analytical procedure based on LC—PDA—ESI (+) —MS for separation, identification and quantification of these adulterants has been developed. To the best of our knowledge we have achieved for the first time in Kuwait, a screening study for presence

of three synthetic PDE-5 inhibitors (S, T and V) in investigated herbal products. Abdel- Hamid study in Kuwait at 2006 [1] has been reported on analysis of S, T and V as adulterants in some herbal products on an individual basis.

EXPERIMENTALS

Chemicals

Standard reference material of sildenafil citrate (purity 98.8%) was received from Pfizer Inc., Groton, CT, USA, tadalafil (purity 99.6%) was received from Eli Lilly, (IN, USA) and vardenafil hydrochloride (purity 99.9%) was received from Bayer Corporation,, Germany. Acetonitrile, methanol (HPLC - grade) and acetic acid analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were analytical grade. A Milli Q2 plus ultra- pure water system from Millipore (Bedford, MA, USA) was used throughout the study to obtain the HPLC –grade water used during the analysis.

Preparation of References Standard Solutions

Stock solution of individual analytes, sildenafil citrate, tadalafil and vardenafil hydrochloride were prepared at concentration of approximately 1mg/ mL in 50:50 (acetonitrile: methanol, v/v) and stored in refrigerator at 4ºC. Working mixture standard solution was prepared fresh daily by diluting stock standards solution with mobile phase consisting of methanol: acetonitrile: 1% acetic acid (20: 20: 60 v/v/v).

Samples

The samples evaluated in this study consisted of a pre-market herbal products (tablets or capsules forms) which have been a subject for registration by KUFDA as a natural herbal products for improving sexual performance for man in the period from 2003 to 2012. Although the samples were acquired from many different sources with different manufacturers, most were labeled to contain a common herb or group of herbs. Some of the more common herbs in this class include, Liquorices root, Wild Yam, Siberian Ginseng, Barbary, wolfberry fruit, Jobs tears seeds, Lilly bulb, Euryale seeds, Chinese yam rhizome and Ginseng root. On the labels of these products, there are some words such as: good supply for energy, improve the body immune system, slow down the aging process in the human bodies and enhance sexual function of man as natural alternatives to the three approved synthetic PDE- 5 inhibitors (S, T and V).

Sample Preparation (Extraction) and Extraction Efficiency (Determination of % Recovery of Analytes)

To determine the content of S, T and V in investigated samples, 3 tablets or 3 whole capsules were weighted; their average weight determined and finally powdered using a mortar and pastel. The weight of tablets or capsules powder equivalent to one dose unite was transferred into 100ml. volumetric flask. Samples were extracted in 50 ml. of methanol: acetonitrile: water (1:3:2, v/v/ v) with sonification for 15min. The flask was completed to 100 ml using the previous mixture of solvent. A representative aliquot was transferred to a centrifuge tube and centrifuged at 3000 rpm for 10 min. A definite volume of the supernatant was diluted to the desired concentration using mobile phase and a portion was transferred to auto sampler vials. For study the analytes recoveries, a portion of the blank herbal product samples (herbal product samples which have been given negative identification for presence of S, T and V were used as a blank sample) triturate equivalent to one dose unit were fortified using a known amount of reference standard at low (L), medium (M) and high levels (H) in triplicate. L, M and H levels presents the amount of the analyte equivalent to 80%, 100% and 120% of the labeled claim [9] for ED treatment using S, T and V as prescribed drugs (25, 20 and 20mg / tablet for S, T and

V respectively). Fortified blank samples were extracted as previously in samples and a portion of diluted supernatant was transferred to autosampler vials. For qualitative and quantitative analysis of S, T and V in extracts of samples and fortified samples, 10ul from autosampler vials were injected three times in LC- PDA – ESI (+) – MS. The percentage of average recovery for each compound and % RSDs were determined.

Qualitative and Quantitative Analysis Using LC—PDA—ESI (+) —MS (Instrument and Conditions)

In this study, qualitative and quantitative analysis have been done using a Liquid Chromatography- Photo Diode Array detector (LC—PDA) coupled to a single quadruple mass spectrometer (MS). The LC—PDA system was consisted of a Waters HPLC Model Alliance 2695 equipped with an autosampler, a column oven and a Waters 996 PDA detector (34 Maple street, Milford, MA, USA). The LC-PDA system was coupled to a single quadruple mass spectrometer equipped with an electrospray ionization source (ESI) (Waters—Micromass ZQ, 34 Maple street, Milford, MA, USA). The analytical column employed was a reversed –phase C18 of 150 mm X 2.1mm and 5um particle size (symmetry 300, Waters, Milford, MA, USA). Analytes separation were carried out by isocratic elution using mobile phase consisting of 1% acetic acid: acetonitrile: methanol (60:20:20, v/v/v), at a flow rate 0.4mL/min under ambient conditions. Analytes detection were done simultaneously by PDA and MS. The electrospray was operated in positive ion mode and the voltage of capillary, extractor, RF lens and cone was set at 4 kV, 6V, 0.5 V and 30 V respectively. The temperature was maintained at 140 C ^{o} and 420 C^{o} for source and desolvation respectively. The gas (N2, purity = 99.999%) flow rate was set at 430 L / hr. Complete system control; data acquisition and data processing were performed using a Mass lynx soft ware. This software has ability for viewing and compare, print MS data and PDA data simultaneously. The full scan mass spectra (Total Ion Current Chromatogram, TIC) and PDA –UV spectra were acquired over a range of m/z 100- 550 amu and 200—400 nm respectively. ESI parameters were optimized using flow injection analysis mode (FIA). For that purpose individual solution from S, T and V was directly infused to MS. Retention times of separated compounds as well on the PDA chromatogram as on the TIC of the MS detector of reference standards and samples were used for preliminary identification of the investigated adulterants. Confirmatory identification was carried out using UV and MS spectral data obtained from reference standards and samples at the same retention time +-5%. In addition selective ion monitoring technique (SIM) was used for more confirmation. For quantitative analysis, LC—PDA— ESI (+) – MS (PDA mode) was used and a calibration curves for S, T and V were constructed. Each analyte was analyzed in its own linearity range.

RESULTS

LC and MS Conditions Optimization

The chromatographic conditions, especially the composition of mobile phase, was optimized through several trials to achieve high sensitivity, high resolution between analytes (especially between S and V) and symmetrical peak shapes for analytes as well as short chromatographic time for analysis using LC — PDA — ESI (+) — MS. In this study separation of S. T and V were accomplished o*n* a symmetry 300 analytical column packed with 5um C18 and mobile phase consisted of methanol: acetonitrile: 1% acetic acid (20:20:60, v/v /v) at a flow rate of 0.4ml/min. under ambient conditions. Detection for the analytes were done using PDA and MS detectors simultaneously. As shown in (Figure 1), it can be concluded that S, T, and V were resolved at the base line using the mobile phase eluted isocratically with resolution values, R = 2.8 and 16.1 between V and S and between S and T respectively. The separated peaks on TIC and PDA

chromatograms have symmetrical shapes with symmetry factor (As) 1.05, 1.02 and 1.01 for S, T and V respectively. The % RSD values for the tR of S, T and V were less than 1.5, indicating the stability of the chromatographic system. In accordance to USP [42] requirements, the quality of chromatographic data obtained about S, T and V are met the acceptable criteria. These demonstrate the suitability of the chromatographic system and its effectiveness for qualitative

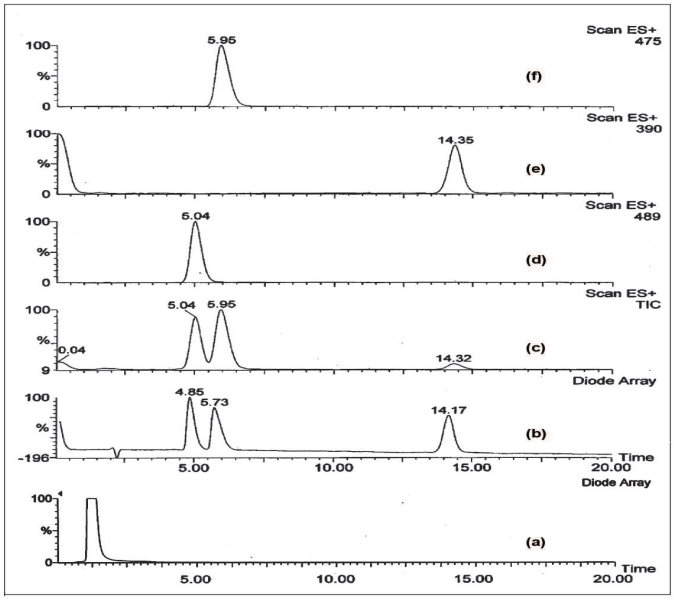

Figure 1: LC—PDA— ESI (+) —— MS Analysis for Extract of Blank Sample and Standard Mixture of S, T and V. a) PDA Chromatogram of Blank Sample Extract. b) PDA Chromatogram for V (tr 4.85 min.), for S (tr = 5.73 min) and for T (tr =14.17 min). c) TIC Chromatogram for V (tr = 5.04 min), for S (tr = 5.95 min) and for T (tr= 14.32 min). d) Extracted Ion Chromatogram for Ion $[M+H]^+$, m/z = 489 amu for V. e) Extracted Ion Chromatogram for Ion $[M + H]^+$, m/z = 390 amu for T. f) Extracted Ion Chromatogram for Ion $[M+H]^+$, m/z =475 amu for S

And quantitative purposes during the course of this study. The MS and UV data obtained from analysis of investigated samles, can be used for positive compound identification by means of comparison to analytical reference standard. The combination of chromatographic retention data, PDA data and MS data that which exhibits both a protonated molecular ion $[M+H]^+$ (corresponding extracted ion chromatogram using SIM technique for ions at m/z =489 amu for V, at m/z =475 amu for S and at m/z =390 amu for T as shown in Figure 1) and other fragment ions can provides a relatively simple method for identification of these compounds. From these data it is clear that LC –PDA – ESI (+) –MS method developed can be serves as a simple and rapid method for separation and detection of S, T and V in investigated samples.

METHODS VALIDATION

The performance of the assay method for S. T and V was evaluated according to UNODC [40] and ICH [41] by estimation of, accuracy (% recovery), linearity range, limit of detection (LOD), limit of quantification (LOQ) and specificity. The accuracy of the method was studied by calculating the average percentage recoveries for S, T and V from fortified blank samples (tablet and capsule forms) at low, medium and high levels. The mean recovery for S, T and V was 95.1-102.2% with % RSD less than 10%. These results about % recovery (accuracy) and %RSD met the acceptable criteria according to UNODC [40] and ICH [41] requirements. This means the developed assay method for analysis of S, T and V in investigated samples showed good accuracy. The calibration data obtained about S, T and V showed there is good linearity for the response of PDA detector was found for all analytes at concentration within the tested range with linear

correlation coefficient higher than 0.9995. The LOD and LOQ were found to be 0.10 and 0.40, 0.20 and 0.55 and 0.10 and 0.51µg /ml for S, T and V respectively. Data about the precision the developed assay method was found to be precise since the % RSD values were less than 15% and 20% for high and low levels of fortification as recommended by UNODC [40] guidelines. These demonstrate the precision of the method and so, its effectiveness for quantitative purposes. The selectivity of the analytical procedures was determined by analysis of blank samples extract using LC — PDA —ESI (+) — MS. No interfering peaks from the endogenous materials of plant constituents of tested herbal products were observed at the retention times of S, T and V as shown in Figure 1. It can be said that under operation conditions of LC —PDA —ESI (+) —MS, S, T and V were adequately resolved from each other and from plant constituents of herbal products.

Additionally using SIM technique provides a very selective detection for analytes in investigated herbal products corresponding to the ions at 475, 489 and 390 amu for S, T and V respectively (Figure 1) (for details about method validation, see [48]). All these validated data of the developed LC — PDA — ESI (+) — MS method for analysis S, T and V in adulterated herbal products were deemed acceptable. The method is precise, selective, accurate and sensitive for the determination of S, T and V in adulterated herbal products.

Analysis of Adulterated Herbal Products

The applicability of the developed and validated method has been demonstrated for analysis the extracts of 485 herbal samples. These samples (a pre market samples) has been a subject for inspection by KUFDA for presence of a well known three synthetic PDE-5 inhibitors S, T and V in the period from 2003 to 2012. Each year the percentage of positive samples identified to be contains individual or mixture of adulterants in relations to the number of sample analyzed were calculated. In an effort to understand the levels of adulteration in investigated herbal samples, S, T and V were quantified. Qualitative and quantitative data are found in Table 1. These data can be summarized in the following: a- The percentage of adulterated samples with at least one of a well known synthetic PDE-5 inhibitors S, T and V ranged between 20.5-84.4% within period of 10 years b- Tadalafil followed by Sildenafil and Vadenafil presents the main adulterants in the investigated samples. c- Beginning from year 2010, two adulterants, S and T were found in the investigated samples with percentage of adulteration 25.5%, 31.5%, and 40.5% for years 2010, 2011 and 2012 respectively. d- As shown it Table 5 quantification of S, T and V in investigated samples revealed that the doses of S, T and V are sufficiently high to be therapeutic in comparing to the approved pharmaceutical dosage forms (S is marketed in 25, 50and 100 mg /tablet while T and V are marketed in 5, 10 and 20 mg /tablet). For many of investigated samples analyzed, the recommended serving size is one unit. Based on the levels per unit dose presented in Table 1, the serving size ensures that these products deliver therapeutic dosage levels of the synthetic PDE-5 inhibitor detected. On the other hand and as shown in Table 1, 2—4% of positive samples for S were found to be contains over therapeutic dose.

DISCUSSIONS

In this study extracts of 485 natural herbal product samples were analysed using the developed and validated analytical method based on LC-PDA –ESI - MS. These samples (a pre market samples and intended to be use for male sexual potency in KUWAIT) has been a subject for inspection by KUFDA for presence the previously mentioned synthetic phosphodiesterase inhibitors type-5 (PDE-5) in the period from 2003 to 2012. The obtained data revealed that the

percentage of adulterated samples with at least one of a well.

Table 1: Synthetic PDE- Inhibitors Were Detected and Quantified as Adulterants in Samples Which Have been a Subject for Inspection by KUFDA as a Natural Herbal Product in the Period from 2003 to 2012

Adulterant	% Sample Positive for Presence of Adulterants (Concentration Range mg/ Dose)									
Year	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
S	20.5 (25-35)	20.7 (25-40)	20.7 (50-75)	21.1 (25-100)	26. 2 (25-150) (.) (•)	27.1 (25-35)	22.5 (25-45)	15.2 (25-130) (..) (••)	11.2 (25-90)	13.5 (50-60)
T	—	30.7 (15-20)	32.7 (8-10)	35.1 (5-8)	40.7 (8-20)	43.1 (10-20)	55.2 (8-20)	40.5 (5-15)	39.5 (5-10)	30.4 (8-20)
V	—	—	10.8 (8-10)	11.2 (10-15)	1.5 (15-20)	—	—	—	—	—
S + T S T T T	— — —	— — —	— — —	— — —	— — —	— — —	— — —	25.5 (25-50) (8-10)	31.5 (25-35) (5-10)	40.5 (25-50) (5-10)
Total % of adulterated samples	20.5	51.4	64.2	71.4	74.4	76.2	77.7	81.2	82.2	84.4
Total % of free adulterated samples	79.5	48.6	35.8	28.6	25.6	23.8	22.3	18.8	17.8	15.6
No. of samples analyzed per year	48	53	43	47	50	48	53	55	45	43

— Samples Free From the Corresponding Adulterant. (.) 2% of the Samples Contains Over Dosage (..) 4% of the Samples Contains Over Dosage

Known synthetic PDE-5 inhibitors (S, T and V) were ranged between 20.5-84.4% within 10 years. Beginning from year 2010, two adulterants, S and T were found in the investigated samples with percentage of adulteration 25.5%, 31.5%, and 40.5% for years 2010, 2011 and 2012 respectively. Adulterations of “natural” herbal products with at least one of the previously mentioned synthetic PDE-5 inhibitors, has been reported since 2001 [1, 2, 5, 8, 9, 13, 16, 17, 18, 19, 28, 30, 33, 35 and 45]. Tadalafil followed by sildenafil presents the main adulterants in the investigated samples. The superiority of Tadalafil over Sildenafil and Vadenafil, it may be due to its less side effects and long post dose [46 and 47]. As mentioned by [46], Tadalafil allows men with erectile dysfunction to have successful intercourse up to 36 hours post dose. So, the designers for adulteration they put these information's about tadalafil in their mind. Quantification of S, T and V revealed that, the doses are sufficiently high to be therapeutic. On the other hand 2-4 % of positive samples for sildenafil were found to contains over therapeutic dose.

As we mentioned previously, all investigated samples were labeled to contains a natural herbal ingredients, including, Liquorices root, Wild Yam, Siberian Ginseng, Barbary, wolfberry fruit, Jobs tears seeds, Lilly bulb, Euryale seeds, Chinese yam rhizome and Ginseng roots, … In addition on the packing of these samples was stated that their ingredient had helped to support male performance.

Neither the patient information nor its packing declared the presence of S, T and V. It is true that undeclared PDE-5 inhibitors in natural herbal products presents a serious health risk (especially the products which contains over therapeutic dose and 2 active ingredient) for patient in Kuwait. So, these products are considered as un-approved and are regulated by KUFDA according to the Pharmaceutical Affairs Law of Kuwait. According to these rules all adulterated herbal products have been cancelled from registration and banned to be used by men in Kuwait**.**

Nowadays, it is well known that the analogues numbers of commercial PDE-5 inhibitors has been increased to about 50 and have been used as adulterants for natural herbal products for improve male sexual function [4, 10, 11, 14, 15, 20, 21, 22, 23, 25, 29, 34, 36,, 38, 39, 43 and 44]. Based on their structural similarities with their synthetic PDE—5 inhibitors (similar biological activities might be expected). These analogues are not FDA approved drugs and no report on their biological activities can be found in published literatures. More importantly the toxicity profile of these analogues is unknown. In Japan, one case of liver function impairment was reported that might be due to use of herbal product containing hydroxyhomosildenafil (one structurally analogues of sildenafil [4]) [11]. So it is dangerous to consume these analogues in adulterated herbal products. So science 2010 our strategies for screening studies were modified to looking for all these analogues besides their ethical ingredients (sildenafil, vardenafil and tadalafil). For that purpose, development of an analytical method in KUFDA laboratories based on LC – ESI - MS /MS are in our consideration.

CONCLUSIONS

In this study analytical procedure depend on LC—PDA—EI (+)- MS was developed, optimized and validated for simultaneous identification and determination of a well known three synthetic phosphodiesterase inhibitors type-5 (PDE-5), sildenafil, tadalafil and vardenafil adulterated in natural herbal products. The method appears specific, precise, sensitive, accurate and relatively simple in both sample preparation and equipments. The procedure providing a very useful tool for rapidly screening (inspection studies) of large amount of natural herbal products samples. Using this analytical procedure the time from sample receipt to structural identification and determination of the adulterant were about 50 minutes. The applicability of the method has been demonstrated for analysis the extracts of 485 natural herbal product samples. These samples (a pre market samples) has been a subject for inspection by KUFDA for presence the previously mentioned synthetic phosphodiesterase inhibitors type-5 (PDE-5) in the period from 2003 to 2012. The obtained data revealed that the percentage of adulterated samples with at least one of a well known synthetic PDE-5 inhibitors ranged between 20.5-84.4% within 10 years. Tadalafil followed by sildenafil presents the main adulterants in the investigated samples. Quantification of S, T and V revealed that, the doses are sufficiently high to be therapeutic. On the other hand 2-4% of positive samples for sildenafil were found to contains over therapeutic dose. According to KUFDA rules all adulterated herbal products have been cancelled from registration and banned to be used by men in Kuwait. Finally it can be concluded that during the last 10 years (2003 - 2012), the KUFDA laboratories have renders a great services for protection of human health in Kuwait against the risk associated with consuming a herbal products were illegally adulterated by these adulterants.

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